Increased Expression of Nucleoside Diphosphate Kinase/nm23 and c-Ha-ras mRNA Is Associated with Spontaneous Lung Metastasis in Rat-transplantable Osteosarcomas

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ABSTRACT

The relationship between expression of nucleoside diphosphate kinase (NDP kinase)/nm23, c-Ha-ras, and c-myc genes and metastatic potential was assessed in rat-transplantable osteosarcoma lines, derived from spontaneous and chemical carcinogen (4-hydroxyamino quinoline-1-oxide)-induced osteosarcomas in Fischer 344/NS1c rats. These osteosarcomas possess metastatic potential and highly metastatic lines spontaneous osteosarcoma-selected lung metastatic lesions and 4-hydroxyaminoquinoline-1-oxide-induced osteosarcoma-selected lung metastatic lesions were respectively established by selectively transplanting lung metastatic lesions. Northern blot analysis revealed that the levels of NDP kinase/nm23 and c-Ha-ras gene expression were increased in line with metastatic ability; thus, transcript levels were remarkably higher in both spontaneous osteosarcoma-selected lung metastatic lesions and 4-hydroxyamino-quinoline-1-oxide-induced osteosarcoma-selected lung metastatic lesions than in their respective low metastatic spontaneous and chemical carcinogen (4-hydroxyamino quinoline-1-oxide)-induced osteosarcoma counterparts. c-myc mRNA expression was observed in all tumor lines, without any correlation with metastatic ability. Southern blot analysis did not show evidence of gross rearrangement or amplification of NDP kinase/nm23, c-Ha-ras, or c-myc genes suggesting regulation of their gene expression at the transcriptional and/or posttranscriptional level. These results indicate that NDP kinase/nm23 and c-Ha-ras might be cooperatively involved in a positive manner in signal transduction processes, especially involving G-protein reactions, responsible for metastasis of rat-transplantable osteosarcomas.

INTRODUCTION

The nm23 gene is a candidate metastasis suppressor, which was originally identified by differential screening of a cDNA library with RNA from low and high metastatic clones of a murine melanoma cell line. Thus, low metastatic K-1735 murine melanoma cell lines were found to contain 10-fold greater amounts of nm23 mRNA than highly metastatic K-1735 cell lines (1). The gene was proposed to encode a NDP kinase based on sequence homology with the Dictyostelium discoideum enzyme (2-4), and other evidence for suppression of metastases. This article must therefore be hereby marked advertisement in accordance with US.C. Section 1734 solely to indicate this fact.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: cDNA, complementary DNA; NDP, nucleoside diphosphate kinase; 4-HAQO, 4-hydroxyamino quinoline-1-oxide; PCR, polymerase chain reaction; SOS, spontaneous osteosarcoma; S-SLM, spontaneous osteosarcoma, selected lung metastatic lesions; COS, chemical carcinogen (4-HAQO)-induced osteosarcoma; C-SLM, 4-HAQO-induced osteosarcoma-selected lung metastatic lesions.

MATERIALS AND METHODS

Tumors and Their Characteristics. The two transplantable osteosarcomas were respectively derived from a SOS which occurred in the left foot bone of a Fischer 344/NS1c rat (purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan) and from a COS detected in the right tibia of a F344 rat. Details regarding tumor induction, tumor transplantation, and the histological and biological characterization for doubling time, osteogenic, and metastatic ability have been previously described (23-25). These two osteosarcomas proved serially transplantable with spontaneous metastasis to the lung. Highly metastatic strains S-SLM and C-SLM were established by serially transplanting lung metastatic lesions into s.c. tissue (Fig. 1). Significant differences in metastatic ability between SOS and S-SLM and between COS and C-SLM were consistently present (Fig. 2; Table 1). All tumors were excised at sacrifice, and portions of tumor nodules were dissected for molecular studies, frozen in liquid nitrogen, and stored at -80°C until use.

Preparation of cDNA Probes. A -0.47-kilobase NDP kinase cDNA fragment was obtained by reverse transcription-PCR amplification. Total RNAs derived from normal rat kidney and heart were reverse transcribed to cDNA using Moloney-murine leukemia virus reverse transcriptase (BRL, Gathersburg, MD) and a 15-mer oligodeoxythymidylic acid primer. The cDNA obtained from the reverse transcription of RNA was subjected to PCR to amplify a 0.47-kilobase fragment of DNA including the rat NDP kinase cDNA sequence (4). The PCR protocol was modified from that provided with the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus, Norwalk, WI). Twenty μl of the reverse transcription mix were mixed with PCR buffer (50 mM KC1-10 mM Tris, pH 8.0-1.5 mM MgCl2-0.01% gelatin), 0.2 mM each of dATP, dCTP, dGTP, dTTP, 0.25 mM each of 3' and 5' 20-mer primers, and 2.5 units of AmpliTag DNA Polymerase (Perkin-Elmer Cetus). The sequences were CAC TTC TGG CCT CAG GA for the 5' primer and ACT CTG GTT TCT CGC GTC TA for the 3' primer. Thirty μl of mineral oil were layered over 200 μl of reaction mixture to prevent evaporation. Samples were subjected to
METASTASIS-ASSOCIATED INCREASED EXPRESSION OF NDP KINASE AND H-ras IN RAT OSTEOSARCOMAS

Spontaneous metastasis
Subcutaneous tumors

Lung
Excised tumor tissues

Tumor tissues

Spontaneous metastasis

Repeat

Lung nodule

Lung nodule

Fig. 1. Schematic illustration of the procedure adopted for selection of tumor cells in metastatic lung lesions. Spontaneous lung metastasis occurred from s.c. transplanted SOS and COS tumors, and highly metastatic lines S-SLM and C-SLM were successfully obtained by subsequent and repeated s.c. transplantation of tumor tissues from lung metastatic nodules.

30–40 cycles, with each cycle consisting of 1 min at 94°C, 2 min at 57°C, and 2 min at 72°C. The amplified segments were extracted after size was verified by electrophoresis in 1% agarose gels containing ethidium bromide and used as the probe for NDP kinase. The mouse c-Ha-ras probe was a 0.46-kilobase EcoRI fragment of Ha-MuSV inserted in plasmid pBS-9 (26). The human c-myc exon3 probe was a 1.5-kilobase EcoRI-ClaI fragment of plasmid pMCE2 (27). These two plasmids were grown in Escherichia coli host HB101. The procedure used for cDNA plasmid-insert preparation was as described by Sambrook et al. (28).

RESULTS

Northern Blot Analysis of NDP Kinase, c-Ha-ras, and c-myc Genes in Rat Osteosarcomas. The NDP kinase cDNA probe obtained from reverse transcription-PCR hybridized with RNAs from normal pancreas, liver, kidney, and heart. The 0.8-kilobase transcripts were expressed in all examined tissues, and the tissue distribution was consistent with that of the rat α-isoform of NDP kinase (4), with strong expression being evident in the kidney and heart, and weak expression in the liver and pancreas (Fig. 3).

Results for NDP kinase, c-Ha-ras, and c-myc mRNAs in lines derived from both spontaneous osteosarcomas and 4-HAQO-induced osteosarcomas are shown in Figs. 4 and 5. The NDP kinase and c-Ha-ras transcript levels were increased in line with metastatic ability, with expression being markedly higher in both highly metastatic variants, S-SLM (Fig. 4, Lanes 8–15) and C-SLM (Fig. 5, Lanes 6–13), in comparison with low metastatic SOS (Fig. 4, Lanes 1–7) and COS (Fig. 5, Lanes 1–5), respectively. No significant differences in NDP kinase expression were detected between s.c. tumors (Figs. 4, Lanes 8–11 and 5, Lanes 6–9) and lung metastatic lesions (Figs. 4, Lanes 12–15 and 5, Lanes 10–13) in the S-SLM and C-SLM cases.

The signal intensities of NDP kinase and c-Ha-ras were quantified using an ATTO densitograph system (ATTO Co., Tokyo, Japan) (Table 2). The NDP kinase intensities were significantly elevated in highly metastatic S-SLM and C-SLM as compared to low metastatic SOS and COS. The c-Ha-ras intensities were increased in both S-SLM and C-SLM, but statistically significant differences were present in only S-SLM case. While c-myc mRNA expression was detected in all tumors, there was no clear relationship to metastatic ability (Figs. 4 and 5).

Southern Blot Analysis of NDP Kinase, c-Ha-ras, and c-myc Genes in Rat Osteosarcomas. The Southern blot analysis did not generate any evidence of major amplification or gross rearrangement of c-Ha-ras, c-myc, and NDP kinase genes in EcoRI digested-genomic DNA samples of rat-transplantable osteosarcomas (Fig. 6).

DISCUSSION

The present study demonstrated a clear relationship between the expression of NDP kinase/nm23 and c-Ha-ras but not c-myc genes and
METASTASIS-ASSOCIATED INCREASED EXPRESSION OF NDP KINASE AND H-ra.s IN RAT OSTEOSARCOMAS

Table 1. Details of the rat-transplantable osteosarcomas investigated

<table>
<thead>
<tr>
<th>Tumor or lesion</th>
<th>Generation (G)</th>
<th>Doubling time (days)</th>
<th>% of metastatic incidence</th>
<th>No. of metastatic nodules/lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOS</td>
<td>G53</td>
<td>2.3</td>
<td>67 (2/3)</td>
<td>4.7 ± 4.1*</td>
</tr>
<tr>
<td>SOS</td>
<td>G55</td>
<td>2.5</td>
<td>100 (3/3)</td>
<td>6.3 ± 3.4</td>
</tr>
<tr>
<td>S-SLM</td>
<td>G26</td>
<td>2.5</td>
<td>100 (2/2)</td>
<td>&gt;300*</td>
</tr>
<tr>
<td>S-SLM</td>
<td>G32</td>
<td>3.1</td>
<td>100 (5/5)</td>
<td>84.8 ± 23.6*</td>
</tr>
<tr>
<td>COS</td>
<td>G38</td>
<td>2.5</td>
<td>100 (5/5)</td>
<td>5.0 ± 4.4</td>
</tr>
<tr>
<td>COS</td>
<td>G43</td>
<td>2.8</td>
<td>67 (2/3)</td>
<td>9.7 ± 7.1</td>
</tr>
<tr>
<td>C-SLM</td>
<td>G26</td>
<td>2.9</td>
<td>100 (3/3)</td>
<td>62.6 ± 15.1*</td>
</tr>
<tr>
<td>C-SLM</td>
<td>G28</td>
<td>3.0</td>
<td>100 (6/6)</td>
<td>38.8 ± 25.6*</td>
</tr>
</tbody>
</table>

* Mean ± SD.

The statistical significant differences determined with the Mann-Whitney test were present between SOS and S-SLM and between COS and C-SLM (P < 0.01, respectively).

Metastatic ability in rat-transplantable osteosarcomas. Thus Northern blot analysis revealed levels of NDP kinase/nm23 and c-Ha-ras mRNA to be increased in line with numbers of metastatic foci generated by osteosarcoma lines. In contrast, c-myc expression did not vary between high and low metastatic lines.

Recently, a number of investigators have studied the association between nm23 expression levels and metastatic phenotype in various human and animal tumors and cell lines (30-35), but the results have been equivocal. Colorectal carcinomas and neuroblastomas were found to exhibit enhanced nm23-H1 mRNA expression in association with tumor progression, in contrast with murine melanoma and breast carcinomas. It was earlier established that NDP kinase/nm23 transcript levels differ among various tissues (4), as confirmed by the present investigation, and a possible tissue-specific involvement of NDP kinase in cellular growth and differentiation has been postulated. Some investigations supported a positive growth link of NDP kinase in Dictostelium discoideum (36) and normal human peripheral blood lymphocytes and leukemia cells (37). Furthermore, recent immunohistochemical studies demonstrated that the level of NDP kinase expression is increased in the proliferating stage and decreased in the differentiating stage in osteoblasts. Our data showing a positive relationship between NDP kinase/nm23 and c-Ha-ras expression and metastatic ability further suggest that these two genes might be involved in accelerating the metastatic process in the case of rat osteosarcomas.

Recent reports have indicated that NDP kinase is transcriptionally regulated in a suppressive fashion by the c-Ha-ras gene (38, 39). The parallel increase in expression of NDP kinase/nm23 and c-Ha-ras mRNA in line with metastatic ability in the present study are, however, in disagreement with the above-mentioned reports indicating suppressive regulation of NDP kinase expression by c-Ha-ras. A possible explanation for this discrepancy could be the differences in the isoform of NDP kinase investigated. Most recently, a second form (β-isoform) rat NDP kinase has been identified, and the rat NDP kinase β gene was demonstrated to have higher homology with human and mouse nm23 gene (40) which showed an inverse relationship with metastatic potential. Furthermore, it has recently been demonstrated

Fig. 3. Northern blot analysis of the tissue distribution of nucleoside diphosphate kinase mRNA. The 0.8-kilobase NDP kinase transcripts were strongly expressed in kidney and heart and weakly expressed in liver and pancreas. This pattern was consistent with that previously reported (4). kb, kilobases.

Fig. 4. Northern blot analysis of nucleoside diphosphate kinase, c-Ha-ras, and c-myc genes in rat spontaneous osteosarcomas. Total cellular RNAs (20 μg) were blotted and hybridized with multiprime labeled cDNAs. Lanes 1–3, SOS G53 s.c. tumors. Lanes 4 and 5, SOS G55 s.c. tumors; Lane 6, SOS G53 lung metastatic lesion; Lane 7, SOS G55 lung metastatic lesion; Lanes 8, S-SLM G26 s.c. tumor; Lanes 9–11, S-SLM G32 s.c. tumors; Lane 12, S-SLM G26 lung metastatic lesion; Lanes 13–15, S-SLM G32 lung metastatic lesions. The loading and transfer of RNA is relatively equal across the various lanes, as determined by using β-actin as an internal control.

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* N. Ishikawa, A. Ishii, and N. Kimura, personal communication.
that allelic loss and mutation of the nm23 gene are associated with metastasis in colorectal cancer (41, 42). Although gross rearrangement was not evident by Southern blot analysis in our tumors, the possibility that the NDP kinase/nm23 and c-Ha-ras genes of these osteosarcomas possessed other alterations such as point mutations might exist. Studies aimed at answering this question are now in progress.

The lack of any evidence of gene amplification of NDP kinase, c-Ha-ras, and c-myc, nevertheless suggests no role for multiple gene copies in genesis of rat osteosarcomas, and that their expression is regulated at the transcriptional and/or posttranscriptional levels.

Metastasis is known to be a complex process, and many specialized characteristics are required for populations to be able to metastasize. We previously found no clear link between the expression of transin, c-fos, and c-jun genes and metastatic potential (24). The present results, on the other hand, revealed a possible involvement of NDP kinase/nm23 and c-Ha-ras genes, alone or in combination, in the metastatic process. The question of whether they are involved cooperatively in signal transduction processes, especially those governing G-proteins and therefore directly influencing expression of the metastatic phenotype, is now undergoing further assessment using our rat-transplantable osteosarcoma system.

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